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CITATION:

Kurachi, Mamoru. Studies on the Biosynthesis of Pyocyanine. (VI) : On the Biochemical Degradation and Resynthesis of Pyocyanine. Bulletin of the Institute for Chemical Research, Kyoto University 1959, 37(2): 73-84

ISSUE DATE:

1959-07-10

URL:

http://hdl.handle.net/2433/75700

Studies on the Biosynthesis of Pyocyanine (VI)

On the Biochemical Degradation and Resynthesis of Pyocyanine

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Received February 4, 1959

In a comparative investigation on the synthesis of pyocyanine between an automatic degradation product of pyocyanine and a biochemical one which had been demonstrated in the cultures of fungi administered pyocyanine, it was found that the degradation product of pyocyanine by fungi could be resynthesized into pyocyanine by resting cells of *Pseudomonas aeruginosa*, while the former could never be so under any condition.

On the other hand, it has been found that fungal strain mentioned above, formed by itself such a product as considered to be a precursor of pyocyanine independently of the administration of pyocyanine. The product derived from pycyanine by fungi has been isolated in crystalline form, but found to be so labile as to be unable to analyze.

INTRODUCTION

At present, the investigation dealing with the mechanism of biosynthesis of pyocyanine has scarcely been reported, although some informations on the effect of amino acid and some organic acids were presented[1~3]. Therefore, the acquirement concerning an intermediate in pyocyanine synthesis has not been furnished to the researcher in this field. In the precedng paper[4], the author has pointed out that according to the cultural condition or to the kind of bacterial strain, a possible intermediate in pyocyanine synthesis might be accumulated in the culture medium. However, it was a very difficult matter to isolate this product in native state. If such a chemically known compound as required specifically for pigmentation can be found out, the research on the present problem will methodologically be advanced. In this sence, the effect of methionine which has been anticipated to play a role of methyl carrier in pyocyanine synthesis system, is of special significance[5]. Possibly, pyocyanine would be synthesized through a roundabout way, because any compound which might have been expected to be a precursor of pyocyanine, judging from a chemical method for this synthesis[6], has wholly been useless for the biosynthesis of this pigment. On the other hand, the oxidative products of pyocyanine by treating with $KMnO_4$, H_2O_2 and other oxidizing agents were also ineffective in this synthesis. After all, it has again come to design to obtain the intermediate product directly from the culture medium of the bacteria. And there has arisen the new idea that a process of biochemical degradation of pyocyanine would suggest the mechanism of its

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biosynthesis as its reversible course, even if the degradation might be of the other organisms.

According to this concept, some attempts have been done, and succeeded in finding out the fact that pyocyanine is broken down by some fungi, and that this degradation product can be resynthesized into pyocyanine by resting cells of the bacteria. The present product has been isolated in crystalline form and some informations on its characteristics have been furnished.

EXPERIMENTAL AND DISCUSSION

Effect of α-Hydroxyphenazine and Other Aromatic Compounds

As shown in Fig. 1, Wrede *et al.*[6] synthesized phenazine nucleus by the condensation of two benzene rings. According to this method, some aromatic compounds have been tested about their effect on pyocyanine formation. The result shown in Table 1 manifested that the intermediary compounds in the chemical synthetic process did not bring any increasing effect on pigmentation, and revealed rather an inhibitory action even in the concentration in which bacterial growth was not affected.

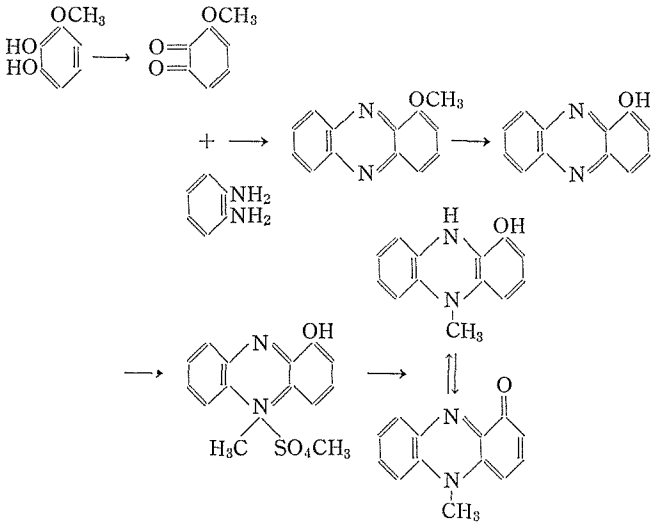

Fig. 1. Method for the chemical synthesis of pyocyanine presented by Wrede *et al.*

Kögl *et al.*[7] have shown that chlororaphin (a quinhydrone-like complex of mol. proportions of phenazine-α-carboxamide and dihydrophenazine-α-carboxamide) formed by *Pseudomonas chlororaphis*, was derived from phenazine-α-carboxylic acid. Referring to this concept, pyocyanine was similarly considered to be synthesized from α-hydroxyphenazine by its methylation. And so, the following two experiments were carried out: (1) 0.002% α-hydroxyphenazine which was prepared by the procedure previously reported[8], was added to the medium composed of 2% glycerol, 0.2% urea, 0.05% $MgSO_4 7H_2O$, 0.025% K_2HPO_4 and 0.0005% $Fe_2(SO_4)_3$, pH 7.4, and incubated with the inoculation of the strains capable and incapable

Table 1. Effect of some aromatic compounds on pyocyanine formation.

Compound (%)	0.1	0.05	0.025	0.010	1.005	0.025
Pyrogallol	*	*	(−) ++	(−) ++	(−) +++	(−) +++
Resorcinol	*	(−) +	(−) ++	(−) ++	(−) +++	(−) +++
Guajacol	*	(−) +	(−) ++	(++) +++	(+) +++	(++) +++
Acetanilide	(−) +	(−) ++	(−) +++	(++) +++	(++) +++	(++) +++
o-Phenylenediamine	*	*	(−) ++	(−) ++	(−) +++	(−) +++
Pyrogallol+*o*-phenylenediamine	*	*	(−) +	(−) ++	(−) +++	(−) +++
DL-Tyrosine	(++) +++	(++) +++	(++) +++	(++) +++	(++) +++	(++) +++
L-Phenylalanine	(++) +++	(++) +++	(++) +++	(++) +++	(++) +++	(++) +++

Basal medium was composed of 2% glycerol, 0.2% urea, 0.05% $MgSO_4 7H_2O$. 0.025% K_2HPO_4 and 0.0005% $Fe_2(SO_4)_3$; pH 7.4.

+++, ++, +, + Express the bacterial cell number of 10^9, 5×10^8, 2×10^8 and 10^8 per ml in a rough estimation, respectively.

(++), (+) The amount of pyocyanine of 0.005% and 0.002% in rough estimation, respectively.

* No multiplication, (−) non pigmentation.

Incubation was performed at 37° for 48 hours.

of pigmentation. (2) The reaction mixture containing 0.1% sodium succinate, 0.01% methionine, 0.05% $MgSO_4 7H_2O$, 0.025% K_2HPO_4, 0.0005% $Fe_2(SO_4)_3$, 0.002% α-hydroxyphenazine and 0.5% acetone powder of the bacteria was incubated at 37° for 24 hours. Against the expectation, as shown in Table 2, α-hydroxy-

Table 2. Effect of α-hydroxyphenazine on pyocyanine formation.

Strains	Incubation period (days)	No. 1 Pyocyanine formed (mg%)	No. 1 α-Hydroxyphenazine remained (mg%)	No. 2 α-Hydroxyphenazine remained (mg%)
B_2	1	4.2	2.0	2.0
	2	11.2	2.0	*
	4	11.8	2.0	*
	6	11.6	2.2	*
C_1	1	—	1.9	2.0
	2	—	*	*
	4	—	*	*
	6	—	2.0	*

No. 1: With the medium containing 2% glycerol, 0.2% urea, 0.05% $MgSO_4 7H_2O$, 0.025% K_2HPO_4, 0.0005% $Fe_2(SO_4)_3$ and 0.002% α-hydroxyphenazine; pH 7.4.

No. 2: Reaction mixture composed of 0.1% sodium succinate, 0.01% methionine, 0.0005% $Fe_2(SO_4)_3$, 0.05% $MgSO_4 7H_2O$, 0.025% K_2HPO_4, 0.002% α-hydroxyphenazine and 0.5% acetone dried cells; pH 7.6. — Non pigmentation. * No measurement.

phenazine did not fluctuate at all in both reaction systems. From the present result, it may be possible to conclude that α-hydroxyphenazine can not be the precursor of pyocyanine in biochemical system. The reason for the above result, of course, must not be attributed to the cell-impermeability of α-hydroxyphenazine. On the other hand, the same result has been shown in the experiment with use of the freezing or grinding cell preparation, so that no consumption of α-hydroxyphenazine might not be ascribable to the impaired enzyme activity of acetone dried cells.

From these facts, it is resulted that α-hydroxyphenazine usually detected in the medium is not the product accumulated as an intermediate in pyocyanine synthesis, but an automatically decomposed product. This may be such a stable substance possessing a less free energy as cannot be reacted as a precursor of pyocyanine in its biosynthesis.

Accordingly, the enzyme catalyzing the methylation of α-hydroxyphenazine cannot also exist in the bacteria. It is, therefore, presumed that pyocyanine is synthesized by the methylation at an earlier reaction step than that of phenazine-ring closure, otherwise by methylating the phenazine derivative other than α-hydroxyphenazine.

Degradation of Pyocyanine by Fungi

In general, pyocyanine added to the bacterial medium is only reversibly reduced and oxidized by the cells without any essential deterioration. However, it has been found that the pyocyanine administered to the medium of fungi such as *Aspergillus oryzae*, *Aspergillus niger* or *Penicillium* sp. was not only reduced reversibly but also further converted irreversibly into a colorless substance *via* a blue colored product. As was described before, these accumulation products were regarded to be a precursor of pyocyanine in biosynthesis system, because they could be converted into pyocyanine by the resting cells prepared not only with the normal strain but with the mutant strain which has lost the ability to form pyocyanine.

The experimental method for the degradation of pyocyanine was as follows : to the medium containing 2% glycerol, 1% peptone, 0.2% urea, 0.05% $MgSO_4 7H_2O$ and 0.05% K_2HPO_4, pyocyanine was added to attain 0.005%, a spore of *Asp. oryzae* was inoculated and incubated at 30° for 4 days. At first, the product had been regarded to be colorless matter, but afterward it was found that this product could be accumulated in the state of the intermediate wearing blue color, according to the kind of fungal strain or to the cultural condition.

As shown in Table 3, this accumulation in the medium is controlled by the concentration of $MgSO_4$ and K_2HPO_4. At an increasing concentration of $MgSO_4$, accumulation product was remarkably decreased in yield, whereas in the case of K_2HPO_4 rather increasing effect was brought about. In regard to the kind of the strain, its accumulation has been shown in the highest amount by the strain of *Asp. oryzae* No. 18, as compared with the other strains (Table 4). It was noted in this case that in the synthetic medium without peptone the degradation of pyocyanine was hardly proceeded probably because of the slow multiplication of

Table 3. Accumulation of blue colored product from pyocyanine.

K_2HPO_4 (%)	0.2	0.05	0.01	0.005	0.001	0.0005	0.0001	Nil
Product	0.462	0.328	0.418	0.300	0.184	0.128	0.186	0.110
Pyocyanine remained (mg%)	—	—	—	1.80	2.08	1.84	1.42	1.64
$MgSO_4 7H_2O$ (%)	0.2	0.05	0.01	0.005	0.001	0.0005	0.0001	Nil
Product	0.062	0.096	0.080	0.014	0.210	0.228	0.280	0.310
Pyocyanine remained (mg%)	—	—	0.42	1.20	1.42	1.28	2.24	2.20

Medium: 2% glycerol, 1% peptone, 0.2% urea, requisite amounts of $MgSO_4$ and of K_2HPO_4, and 0.005% pyocyanine.

For the test of K_2HPO_4, concentration of $MgSO_4 7H_2O$ was kept to 0.025%, and for $MgSO_4$; K_2HPO_4, to 0.025%. Incubation was performed at 30° for 4 days. The product accumulated was expressed in relative amount by measuring the value of optical density at 660 mμ.

Strain used was *Asp. oryzae* No. 31.

Table 4. Degradation of pyocyanine by fungi.

Strains	*Aspergillus oryzae*						*Penicillium* sp.		
	No. 27	No. 29	No. 30	No. 31	No. 34	No. 18	No. 24	No. 5	No. 2
Pyocyanine remained (%)	Trac	0.001	0.002	0.001	0.0008	0.001	0.003	0.002	0.002
Blue colored product	0.072	0.028	0.124	0.168	0.082	0.396	—	0.062	—

Medium was composed of 2% glycerol, 1% peptone, 0.2% urea, 0.05% $MgSO_4 7H_2O$, 0.05% K_2HPO_4 and 0.005% pyocyanine.

The expression of the product was the same as in Table 3.

fungal mycelium, and that in the experiment with use of cell homogenate, the result was observed to be far inferior in yield of the intermediate to that with the intact mycelium.

Effect of the product derived from pyocyanine

The experiment on the effect of the product derived from pyocyanine has been done by the following procedure: The peptone media containing 0.005% pyocyanine, inoculated respectively with Strains No. 18 and No. 29 were incubated at 30° for 4 days. The cultured solution of No. 18, from which mycelium was eliminated, was treated with chloroform to remove the pyocyanine remained and concentrated to add to the medium of the bacteria.

Separately, the above cultured solution, after saturating with ammonium sulfate, was extracted with butanol and the butanol layer was transferred into a small amount of aqueous solution by shaking with excess of ethyl ether. On the other hand, from the residual part obtained above, aqueous solution was prepared by the further extraction of the acidified solution with butanol. In parallel

with the above experiments, with the cultured solution of Strain No. 29 which was almost colorless, differing from No. 18, the same extracting procedure was performed. These samples, followed by sterilization through the addition of chloroform, were added to the synthetic medium prepared for pyocyanine formation, inoculated with Strains C_1 incapable of pigmentation and Bd showing a less capacity for pigmentation, after aspirating the medium to remove the chloroform involved and incubated at 37° for 48 hours. Results are shown in Table 5.

Table 5. Pyocyanine formation by mutant strain.

Strains	Samples				Control
	No. 1	No. 2	No. 3	No. 4	
C_1	0.003	0.004	0.004	0.003	—
Bd	0.008	0.009	0.010	0.006	0.002

No. 1 contains the degradation product of pyocyanine by *Asp. oryzae* No. 18 ; No. 2, the butanol extract of No. 1 ; No. 3, the residue of the butanol extract of No. 1 ; No. 4, the product from pyocyanine by *Asp. oryzae* No. 29. Basal medium was the same as in Table 1. Incubation, at 37° for 48 hours. Pyocyanine was expressed in %.

It was demonstrated that the degradation products of pyocyanine by fungi might be resynthesized into pyocyanine, differing from the case of α-hydroxyphenazine and other intermediates in chemical synthetic process. It has been believed by the author that possible intermediates in pyocyanine synthesis may be accumulated in the medium of the mutant strain, or of the normal strain incubated in the presence of inhibitory agent[4]. If the present result is put together, success in the formation of pyocyanine by the mutant strain will more support the concept mentioned abve.

It should be noted that both of the products from pyocyanine by Strains No. 18 and No. 29 revealed the same effect on the synthesis of pyocyanine. The effect of the residual part through the butanol extraction of the blue colored product suggests that the colorless product is also an intermediate in the degradation of pyocyanine, accumulated at a further step.

Since a fluctuation of the colorless product was difficult to trace, the following test has been tried : the peptone media in which 0.005% and 0.002% pyocyanines

Table 6. Effect of the product derived from pyocyanine by fungi on pyocyanine formation.

Original amount of pyocyanine (%)	Pyocyanine remained (%)	Pyocyanine formed (%)	Sum of pyocyanines (%)
0.0020	0.0006	0.0040	0.0046
0.0050	0.0008	0.0060	0.0068

Fungal strain, *Asp. oryzae* No. 29 was incubated at 30° for 3 days.

Bacterial strain, C_1 was incubated at 37° for 48 hours. Basal medium of the bacteria was the same as in Table 1. Fungal medium was the same as in Table 4.

were contained respectively, prepared by the procedure mentioned before, were inoculated with Strain No. 29 and incubated at 30° for 3 days. By the extraction of the cultured solution with chloroform, the amount of pyocyanine remained was determined and its residual part followed by the butanol extraction procedure was provided to the medium of the bacterial strain, C_1. The sum of pyocyanines remained and formed was compared with its original amount (Table 6). Strange to say, not only the pyocyanine appeared did not agree in amount with the one disappeared but also the former rather exceeded the latter. From these results, there has arisen a question that fungi might, by themselves, form the metabolic product expected to become the precursor of pyocyanine.

Effect of Metabolic Product of Fungi

The following experiment was carried out to answer the above question : the peptone media as mentioned before, inoculated with the fungal strains, were incubated without administration of pyocyanine. The cultured solutions of each strain were treated with butanol, including the peptone medium which was not inoculated to assign as a control. The synthetic medium containing the above extract, inoculated with Strain C_1, was incubated at 37° for 48 hours. As shown in Table 7, from the cultured solutions of every strain of fungi, pyocyanine formation was brought about.

Table 7. Effect of the product of fungi on pyocyanine formation.

Strains	*Aspergillus oryzae*					*Penicillium* sp.			Control
	No. 29	No. 30	No. 31	No. 34	No. 18	No. 24	No. 25	No. 2	
Pyocyanine formed (%)	0.001	0.003	0.002	0.004	0.004	0.005	0.003	trace	—

Basal medium of the bacteria was the same as in Table 1.
Incubation temperature and period in fungi were the same as in Table 5.
Bacteiral strain C_1 was incubated at 37° for 48 hours.

It was a surprising event that pyocyanine could be produced by the mutant strain which lost the ability to form pyocyanine, depending on the metabolite of the other organism. From these facts, it has now come to be very obscure to distinguish whether the formation of pyocyanine by mutant strain should be ascribable to the product derived from pyocyanine, or to that of fungi themselves. However the blue colored product has strictly maintained its meaning, because this substance can be converted to pyocyanine under the enzymatic condition using the resting cells, and can easily be caught in its fluctuation, being compared with forming pyocyanine, as will be illustrated later.

But, at any rate, why could the other organism independent of pigmentation produce such a metabolite as regarded to be the precursor of pyocyanine?

For the explanation of this question, the following discussions will be brought up :

1. The capacity of fungal strain for the degradation of pyocyanine may be induced by the administration of pyocyanine, which may be maintained for

several generations, and therefore the product regarded as an intermediate in pyocyanine synthesis can be formed by the reverse reaction of its decomposing process, even in the absence of pyocyanine.

2. These capacities of fungi may rather be due to the constitutive enzyme system, and the substances similar to the precursor of pyocyanine can, by themselves, be produced as a metabolite of fungi according to the reverse mechanism of the degradation.

Based on the experimental consideration, the concept described in Clause 2 will be supported, because however many times the generation culture might be repeated, or whatever a virgin strain might be supplied to the experiment, the formation of the effective product was always revealed in the absence of pyocyanine. There should be a causal relationship between the abilities to decompose pyocyanine and to form the metabolite regarded as a precursor of pyocyanine.

Synthesis of Pyocyanine by Resting Cells

According to the method mentioned before, from the cultured solution of *Asp. oryzae* No. 18 incubated in the presence and absence of pyocyanine, the following three fractions were obtained: (1) the blue colored fraction, (2) the residual part of (1), (3) the extract of the medium incubated in the absence of pyocyanine.

These fractions were added to the bacterial medium consisting of 0.1% sodium succinate, 0.025% K_2HPO_4 and 0.025% $MgSO_47H_2O$, pH 7.6.

The sampling cells were cultivated as follows: 2% glycerol, 1% peptone, 0.2% urea, 0.025% $MgSO_47H_2O$, 0.025% K_2HPO_4 and 3% agar were dissolved in 100 ml of distilled water, adjusted to pH 7.4 and pasteurized. To 20 ml of the medium taken into an Erlenmeyer flask of 300 ml in capacity, was added 0.1 g of charcoal and then placed into a Petri dish of 15 cm in diameter, after shaking in order to mix uniformly. And the liquid agar medium was put over the solidified medium in thickness of 1 mm. The pyocyanine formed in the medium thus prepared was diffused so as to be adsorbed on charcoal, and the pyocyanine-free bacterial cells could be harvested. From the cultures with an ordinary medium,

Table 8. Pyocyanine synthesis by resting cells.

Cells	Fractions No. 1	No. 2	No. 3
No. 1	0.006	—	—
No. 2	—	—	—
No. 3	0.004	—	—

Fractions: No. 1, blue colored product derived from pyocyanine; No. 2, the residual part of the butanol extract of No. 1; No. 3, the extract of the medium incubated in the absence of pyocyanine. Every sample was prepared from the cultured solution of *Asp. oryzae* No. 18.

Resting cells of bacteria: No. 1, cells of Strain B_1 harvested from the agar medium containing 2% glycerol, 0.2% urea, 0.05% $MgSO_47H_2O$, 0.025% K_2HPO_4 and 0.0005% $Fe_2(SO_4)_3$; No. 2, cells of Strain B_1 grown on the excess of phosphate; No. 3, cells of Strain C_1 which has lost the ability for pigmentation.

Sample cells were harvested after 24 hours' incubation, respectively.

pyocyanine-free cells could never be obtained even when washing might be repeated so many times. The cells harvested from the plate culture incubated at 37° for 24 hours were suspended in distilled water to be on the spacious surface, allowed to stand for 12 hours at 37°, centrifuged and resuspended in the medium mentioned above at a level of 0.2 g of wet cells per 10 ml of the solution. In parallel with this experiment, pyocyanine-free cell preparation was obtained from the cultures of the bacteria grown on the agar medium containing K_2HPO_4 in the concentration higher than 0.1%. On the other hand, the resting cells of the mutant strain, C_1 were prepared from a plate culture of the normal medium. These results are shown in Table 8.

The formation of pyocyanine by resting cells has taken place with success, although the samples Nos. 2 and 3 were negative in result. It was noted that pyocyanine formation could be revealed even by the mutant strain incapable of pigmentation, and that resynthesizing reaction into pyocyanine could proceed so readily as to be recognizable within 5 hours after the incubation. It is a noticeable fact that the cells harvested from the medium of excess of phosphate did not reveal any pyocyanine formation, even when a low level of phosphate was kept in the incubation process.

Isolation of the Blue Colored Product

The following medium was chosen to increase the yield of the product: 2% glycerol, 1% peptone, 0.2% urea, 0.0001% $MgSO_4 7H_2O$ and 0.1% K_2HPO_4 were dissolved in 100 ml of distilled water, pH 7.4, and pyocyanine was added to the sterilized medium to attain 0.005% and then incubated at 30° for 4 days, with the inoculation of *Asp. oryzae* No. 18. The cultured solution free from fungal mycelium was extracted with chloroform to eliminate pyocyanine remained, aspirated to drive out the chloroform involved, saturated with ammonium sulfate and extracted with butanol. The butanol layer was transferred into a small amount of aqueous solution by shaking with the excess of ethyl ether. The aqueous solution, followed by aspirating the ethyl ether, was again saturated with ammonium sulfate and further extracted with butanol. The concentrated sample solution could be obtained by repeating the above procedure without aspirating evaporation. When aqueous or butanol solution was concentrated under reducing pressure through air current, it was completely broken down into a green colored substance after 30 minutes even at room temperature. It was observed that alumina, silica gel, starch and other major adsorbents could not be employed for column chromatography, because the decomposition was caused during the development of the sample, especially in the case of starch, the sample was wholly broken down within 1 hour. Similarly, ion exchange resin was unsuitable for this purpose. When paper chromatography was applied to this experiment, sample was completely decomposed at the time of its dryness.

In spite of such labile property as described above, the employment of $CaCO_3$ for the adsorption was found to bring a success in obtaining this substance in crystalline form, according to the procedure as mentioned below: the concentrated butanol solution was kneaded with $CaCO_3$, placed on column of $CaCO_3$ and deve-

loped descendingly with the solvent, *n*-butanol-ethanol-water (1 : 1 : 2). The blue colored spot in column was taken out, eluted with distilled water, and concentrated into a small amount of butanol solution, through the procedure already described. To the extremely concentrated butanol solution, ether was added so as to become cloudy and blue needles were formed by keeping in ice box (Fig. 2).

Fig. 2. Crystals of blue colored product derived from pyocyanine by fungi (×150).

Characteristics of the Blue Colored Product

In Table 9 and Fig. 3, is shown an outline of the property of this substance. In the cultured solution of fungi, this product is nearly colorless, indicating a reducible property as in the case of pyocyanine, and its oxidized form bears such

Table 9. Property of the blue colored product derived from pyocyanine.

	Solubility					
	Chloroform	Ether	Butanol	Methanol	Ethanol	Water
Pyocyanine	+	Slightly	+	+	+	+
Product	−	−	Slightly	−	−	+

	Color		Absorption maxima	
	Alkaline or neutral	Acidic	Alkaline or neutral	Acidic
Pyocyanine	Blue	Red	690 mμ	520 mμ
Product	Blue	Yellowish brown	660 mμ	400 mμ

	Oxido-reduction by cells	Rf value	
		Ethanol-water (7 : 3)	*n*-Butanol saturated with water
Pyocyanine	+	0.61	0.36
Product	+	0.67	−

	Stability			
	Alkali	Acid	Heat	Molecular oxygen
Pyocyainne	Labile	Stable	Labile	Labile
Product	Labile	Very labile	Very labile	Labile

a resemblance, in appearance, to pyocyanine that they can hardly be distinguished from each other. However, differing from pyocyanine, this substance is hardly soluble in any organic solvent, although this is more or less soluble in butanol. On the contrary, it is extremely soluble in water, so that the butanol extraction of its aqueous solution can never be performed without saturation with ammonium sulfate. Against heat, molecular oxygen or acid, it is very unstable, particularly to heat, so labile as to be decomposed completely in a moment. Blue color in neutral or alkaline solution changes to yellowish brown in acid solution and becomes irreversible after several hours even in a slightly acidic state, owing to its decomposition.

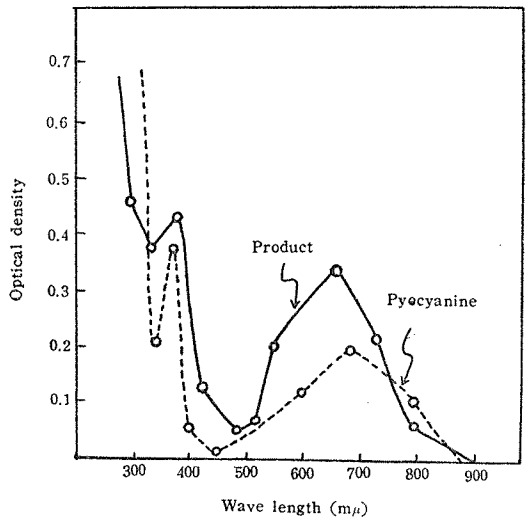

Fig. 3. Absorption spectrum of blue colored product derived from pyocyanine by fungi.

On the other hand, when its aqueous solution was allowed to stand for several days at room temperature, being laid with butanol, the butanol layer would become pink on account of its conversion into the butanol-soluble substance, which appeared to be different from the case of heat or acid effect. Thus, this product is extremely unstable and a stable derivative has not been found out, so that it has not come to be analyzed as yet.

SUMMARY

1. It has been demonstrated that in biosynthesis of pyocyanine, the enzyme catalyzing the methylation of α-hydroxyphenazine cannot exist in this system, and that the biosynthesis of pyocyanine may be performed through a roundabout way differing from the case of the chemical synthetic process presented by Wrede *et al.*

2. It was found that pyocyanine could be degenerated by fungi such as *Aspergillus oryzae*, *Aspergillus niger* or *Penicillium* sp., and that the degradation product could be resynthesized into pyocyanine by the bacteria, suggesting that

this may be one of the precursors of pyocyanine, and the decomposing process of pyocyanine in the fungi may be a reverse course of its synthesis system in the bacteria.

3. Degradation product of pyocyanine has been isolated in crystalline form and shown to be of so labile property as to be unable to analyze.

4. The fungi capable of decomposing pyocyanine were found to produce by themselves such a metabolite as regarded to be a precursor of pyocyanine, independently of the administration of pyocyanine.

The autor wishes to express his hearty appreciation to Prof. H. Katagiri for his generous direction throughout this work.

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